

## CLAIMS

We claim:

1. A method of molecular cloning, wherein a nucleic acid insert molecule is covalently joined to first and second nucleic acid flanking molecules to form a ligated molecule, the method comprising:

(A) incubating said insert molecule and said flanking molecules, wherein each end of said insert molecule comprises a 5'-hydroxyl group, and wherein one end only of each of said first and second flanking molecules comprises a covalently bound topoisomerase polypeptide, under conditions which permit their covalent joining to form a ligated molecule comprising a said insert molecule positioned between a said first and a said second flanking molecule; and

(B) transforming the covalently joined insert molecule of step (a) into a host cell to obtain transformants.

2. A method of covalently joining a nucleic acid insert molecule to first and second nucleic acid flanking molecules to form a ligated molecule, the method comprising

(A) incubating:

said insert molecule, wherein one end of said insert molecule comprises a 5'-hydroxyl group and the other end comprises a 5'-phosphate group, and

a said first flanking molecule, wherein one end only of said first flanking molecule comprises a covalently bound topoisomerase polypeptide, under conditions which

permit their covalent joining to form a ligated nucleic acid comprising a said insert molecule positioned adjacent to a said first flanking molecule,

(B) incubating a said ligated nucleic acid of step (A) with phosphatase under conditions which permit removal of a 5'-phosphate group from said ligated nucleic acid; and

(C) incubating a product of step (B) with a said second flanking molecule, wherein one end only of said second flanking molecule comprises a covalently bound topoisomerase polypeptide, under conditions which permit covalent joining to form a ligated molecule comprising a said insert molecule positioned between a said first and a said second flanking molecule.

3. A method of covalently joining a nucleic acid insert molecule to first and second nucleic acid flanking molecules to form a ligated molecule, the method comprising

incubating:

said insert molecule and said flanking molecules, wherein one end of said insert molecule comprises a 5'-hydroxyl group and the other end comprises a 5'-phosphate group, wherein one end only of said first flanking molecule comprises a covalently bound topoisomerase polypeptide and wherein one end of said second flanking molecule comprises a ligase substrate site, under conditions which permit their covalent joining to form a ligated molecule comprising a said insert molecule positioned between a said first and a said second flanking molecule.

4. The method of claims 1, 2, or 3 wherein a said first and a said second nucleic acid flanking molecules comprise a left and a right vector arm, respectively, such that a said insert molecule is flanked by a said left vector arm and a said right vector arm.

5. The method of claim 4, wherein said left and right vector arms each comprise a free end that is not joined to an insert molecule, said method further comprising the step of:

joining the free ends of said vector arms to each other by a method selected from the group consisting of nucleic acid ligase mediated ligation, complementary sequence annealing, topoisomerase mediated ligation, *in vitro* site-specific recombination, *in vivo* site-specific recombination, and *in vivo* homologous recombination.

6. A method of molecular cloning comprising:

(A) incubating a nucleic acid insert molecule comprising a 5'-hydroxyl group at one end and a 5'-phosphate at the other end, and a linear cloning vector, wherein the linear cloning vector comprises a covalently bound topoisomerase polypeptide at one end only and a ligation substrate site at the other end, under conditions sufficient for their covalent joining to form a ligated circular vector comprising said linear cloning vector and said insert molecule; and

(B) transforming the ligated circular vector of step (A) into a host cell to obtain transformants.

7. A method for molecular cloning comprising:

(A) incubating a nucleic acid insert molecule, wherein each end of said insert molecule comprises a 5'-hydroxyl group, and a first and a second linear arm wherein one end only of each of said first and second linear arms comprises a covalently bound topoisomerase

and the other end comprises a cloning substrate site, under conditions sufficient for their covalent joining to form a ligated molecule comprising a said insert molecule positioned between a said first and a said second linear arm; and

(B) transforming the ligated insert molecule of step (A) into a host cell to obtain transformants.

8. The method of claim 7 wherein said cloning substrate site is selected from the group consisting of a *cos* site, a LIC site, and a loxP site.

9. The method of claim 7 wherein said cloning substrate site is loxP and wherein said incubating step further comprises incubating *in vitro* said ligated molecule with a Cre recombinase and a circular plasmid comprising a loxP site, under conditions sufficient for site-specific recombination to form a circular plasmid comprising said ligated molecule, wherein said circular plasmid comprising said ligated molecule is transformed into a host cell.

10. The method of claim 7 wherein said cloning substrate site is loxP and wherein said transforming step further comprises transforming said ligated molecule into a host cell comprising a circular plasmid comprising a loxP site, wherein said cell expresses Cre recombinase, under conditions sufficient for site-specific recombination to form a circular plasmid comprising said ligated molecule within said cell.

11. The method of claim 7 wherein said cloning substrate site is a site for homologous recombination with a circular plasmid vector, wherein said transforming step further comprises transforming said ligated molecule into a host cell comprising a circular plasmid vector, wherein said circular plasmid vector comprises a site for homologous recombination with said ligated

molecule, wherein said host cell is recA+, under conditions sufficient for homologous recombination to form a circular plasmid comprising said ligated molecule within said host cell.

12. The method of claim 7 wherein said first linear arm comprises a left lambda arm comprising at one end only a covalently bound topoisomerase, and wherein the second linear arm comprises a right lambda arm comprising at one end only a covalently bound topoisomerase.

13. A method for molecular cloning comprising:

(A) incubating a nucleic acid insert molecule, wherein one end of said insert molecule comprises a 5'-hydroxyl group and the other end comprises a 5'-phosphate group, and a first linear arm, wherein one end only of said first linear arm comprises a covalently bound topoisomerase polypeptide and the other end comprises a cloning substrate site, under conditions which permit their covalent joining to form a ligated insert/first linear arm molecule;

(B) incubating a said ligated insert/first linear arm molecule of step (A) with phosphatase under conditions which permit removal of a 5'-phosphate group to form a product which comprises a said ligated insert/first linear arm molecule that lacks a 5' phosphate; and

(C) incubating said product of step (B) with a second linear vector arm, wherein one end only of said second linear vector arm comprises a covalently bound topoisomerase polypeptide and the other end comprises a cloning substrate site, under conditions which permit covalent joining to form a ligated molecule comprising a said insert molecule positioned between a said first and a said second linear vector arm;

(D) transforming the ligated molecule of step (C) into a host cell to obtain transformants.

14. The method of claim 13 wherein said cloning substrate site is selected from the group consisting of a *cos* site, a LIC site, and a loxP site.

15. The method of claim 13 wherein said cloning substrate site is loxP and wherein said ligated molecule is further incubated *in vitro* with a Cre recombinase and a circular plasmid comprising a loxP site, under conditions sufficient for site-specific recombination to form a circular plasmid comprising said ligated molecule, wherein said circular plasmid comprising said ligated molecule is transformed into a host cell.

16. The method of claim 13 wherein said cloning substrate site is loxP and wherein said transforming step further comprises transforming said ligated molecule into a host cell comprising a circular plasmid comprising a loxP site, wherein said cell expresses Cre recombinase, under conditions sufficient for site-specific recombination to form a circular plasmid comprising said ligated molecule within said cell.

17. The method of claim 13 wherein said cloning substrate site is a site for homologous recombination with a circular plasmid vector, wherein said transforming step further comprises transforming said ligated molecule into a host cell comprising a circular plasmid vector, wherein said circular plasmid vector comprises a site for homologous recombination, wherein said host cell is *recA*<sup>+</sup>, under conditions sufficient for homologous recombination to form a circular plasmid comprising said ligated molecule within said host cell.

18. The method of claim 13 wherein said first linear arm comprises a left lambda arm comprising a covalently bound topoisomerase polypeptide at one end, and wherein the second linear arm comprises a right lambda arm comprising a covalently bound topoisomerase polypeptide at one end.

19. A method for molecular cloning comprising:

(A) incubating:

a nucleic acid insert molecule, wherein one end of said insert molecule comprises a 5'-hydroxyl group and the other end comprises a 5'-phosphate group;

a first linear arm, wherein one end only of said first linear arm comprises a covalently bound topoisomerase polypeptide and the other end comprises a cloning substrate site; and

a second linear arm, wherein one end of second linear arm comprises a ligase substrate site and the other end comprises a cloning substrate site, under conditions which permit their covalent joining to form a ligated molecule comprising a said insert molecule positioned between a said first and a said second linear arm;

(B) transforming the ligated molecule of step (A) into a host cell to obtain transformants.

20. The method of claim 19 wherein said cloning substrate site is selected from the group consisting of a *cos* site, a LIC site, and a loxP site.

21. The method of claim 19 wherein said cloning substrate site is loxP and wherein said incubating step further comprises incubating *in vitro* said ligated molecule with a Cre recombinase and a circular plasmid comprising a loxP site, under conditions sufficient for site-specific recombination to form a circular plasmid comprising said ligated molecule, wherein said circular plasmid comprising said ligated molecule is transformed into a host cell.

22. The method of claim 19 wherein said cloning substrate site is loxP and wherein said transforming step further comprises transforming said ligated molecule into a host cell comprising a circular plasmid comprising a loxP site, wherein said cell expresses Cre recombinase, under conditions sufficient for site-specific recombination to form a circular plasmid comprising said ligated molecule within said cell.

23. The method of claim 19 wherein said cloning substrate site is a site for homologous recombination with a circular plasmid vector, wherein said transforming step further comprises transforming said ligated molecule into a host cell comprising a circular plasmid vector, wherein said circular plasmid vector comprises a site for homologous recombination, wherein said host cell is recA+, under conditions sufficient for homologous recombination to form a circular plasmid comprising said ligated molecule within said host cell.

24. The method of claim 19 wherein said first linear arm comprises a left lambda arm comprising at one end only a covalently bound topoisomerase polypeptide, and wherein the second linear arm comprises a right lambda arm comprising at one end a ligase substrate site.